The malarial parasite *Plasmodium falciparum* imports the human protein peroxiredoxin 2 for peroxide detoxification

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Coevolution of the malarial parasite and its human host has resulted in a complex network of interactions contributing to the homeodynamics of the host-parasite unit. As a rapidly growing and multiplying organism, *Plasmodium falciparum* depends on an adequate antioxidant defense system that is efficient despite the absence of genuine catalase and glutathione peroxidase. Using different experimental approaches, we demonstrate that *P. falciparum* imports the human redox-active protein peroxiredoxin 2 (hPrx-2, hTPx1) into its cytosol. As shown by confocal microscopy and immunogold electron microscopy, hPrx-2 is also present in the Maurer's clefts, organelles that are described as being involved in parasite protein export. Enzyme kinetic analyses prove that hPrx-2 accepts *Plasmodium* cytosolic thioredoxin 1 as a reducing substrate. hPrx-2 accounts for roughly 50% of thioredoxin peroxidase activity in parasite extracts, thus indicating a functional role of hPrx-2 as an enzymatic scavenger of peroxides in the parasite. Under chloroquine treatment, a drug promoting oxidative stress, the abundance of hPrx-2 in the parasite increases significantly. *P. falciparum* has adapted to adopt the hPrx-2, thereby using the host protein for its own purposes.

antioxidant defense | protein import | redox metabolism | thioredoxin | Maurer's clefts

To maintain adequate antioxidant defense throughout its complex life cycle, the malarial parasite *Plasmodium falciparum* has developed an elaborate redox system. More than 20 proteins assemble this network, comprising a thioredoxin and a glutathione system, as well as superoxide dismutases and low molecular weight antioxidants (1). The absence of catalase and a genuine glutathione peroxidase, as well as the presence of 4 peroxiredoxins (Prx) that are mainly thioredoxin-dependent, suggest that hydroperoxide detoxification in *P. falciparum* largely depends on the thioredoxin system (2). Thioredoxin-dependent Prx (TPx) are important components of eukaryotic redox systems. Because of high intracellular concentrations, some Prx are involved in peroxide detoxification (3). In eukaryotes, Prx also have regulatory and signaling functions associated with oxidative challenge (4). Our data provide previously undocumented insights into the complex host-parasite interactions, as we show that the human antioxidant protein hPrx-2 is imported from the host cell to the cytosol of *P. falciparum* and that it is enzymatically active with cytosolic parasite-derived redox partners. Furthermore, we provide proof for a colocalization of hPrx-2 with Maurer's clefts (MCs). These parasite-derived membranous structures bud from the parasitophorous vacuolar membrane (PVM) and extend through the RBC cytoplasm to its plasma membrane. These organelles have so far been shown to be involved in parasite protein export (5).

Results

hPrx-2 Is Present in Protein Extracts of *P. falciparum*. We have studied the proteome of 4 *P. falciparum* strains (3D7, HB3, K1, and Dd2) using 2-dimensional electrophoresis (2DE). Our proteomic analyses reproducibly showed the presence of the human protein hPrx-2 in parasite extracts. The protein was unambiguously identified by mass spectrometric (MS) peptide mass fingerprinting (PMF) in 6 protein spots on the gels, as seen in Spots 1 to 6 in Fig. 1*A*, Table S1, and Fig. S1. The sequences of the hPrx-2 spots covered by PMF are summarized in Fig. S1 and in the *SI Text*. The percentage of the covered sequence does not essentially differ, and the sequences covered by higher molecular weight (putative full-length hPrx-2) and putative lower molecular weight spots both contain or embrace the active site Cys-51. The C-terminus of the protein has, however, not been identified in the protein spots. Interestingly, the only plasmodial Prx that was detected on 2DE gels was *Pf*-1-Cys-Prx (see Spot 7, Fig. 1*A*). According to the 2DE data, hPrx-2 is ≈10 to 12 times more abundant than *Pf*-1-Cys-Prx in parasite extracts.

The presence of hPrx-2 in parasite extracts was confirmed with immunoblots, showing that hPrx-2 runs in 3 bands at ≈15, 17, and 22 kDa. These correlate well with the apparent molecular masses of the 6 detected hPrx-2 spots on the 2DE gels (Fig. 1*B*). The antibody used in the immunoblots is specific for hPrx-2 and does not cross-react with either of the 4 parasitic Prx (*Pf*TPx1, *Pf*TPx2, *Pf*-1-Cys-Prx, and *Pf*AOP) (Fig. S2).

hPrx-2 Is Enriched in Relation to Hemoglobin in Protein Extracts of *P. falciparum*. To determine if the presence of hPrx-2 in parasite extracts is a result of intraparasitic hPrx-2 or if it might be caused by preparative contamination with host-cell material, we analyzed the relative abundance of hPrx-2 compared to the RBC cytosolic protein hemoglobin both in the parasite preparation and the host cell. The method that enabled detection of both proteins in the same sample and experiment is liquid chromatographic (LC) separation coupled to highly accurate MS (6) (details in the *SI Text*). Hb was the protein chosen for comparison as it is the best-studied human protein in the host-parasite

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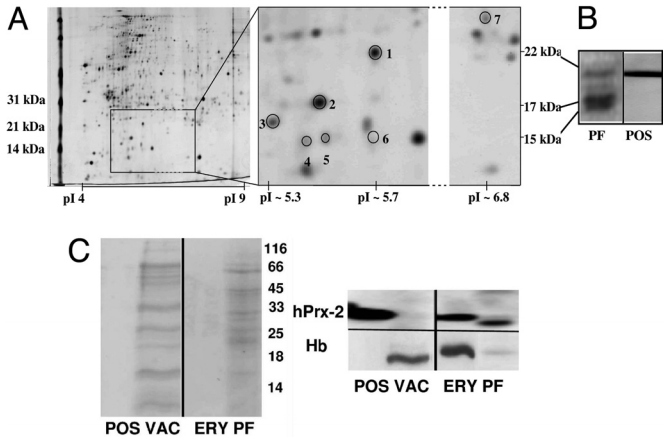

Fig. 1. hPrx-2 is present in *P. falciparum* extract and is not detected in FV preparations. (*A*) Two-dimensional electrophoresis gels of *P. falciparum* trophozoite extracts. Six protein spots were identified as hPrx-2. Spot 7 corresponds to the parasitic enzyme *Pf*TPx-1. (*B*) hPrx-2 immunoblot of *P. falciparum* extract using a polyclonal antibody recognizing the peptide L^{103}LADVTRRLSED^{114} of the human protein. The blot shows the recombinant hPrx-2 positive control (POS) on the right and the presence of hPrx-2 at 3 molecular weight bands in parasite extract (PF). (*C*) hPrx-2 immunoblot of food vacuoles (FVs) shows enrichment of hPrx-2 in parasite lysate. Thirty micrograms of parasite and FV extract was loaded per lane, 0.4 μg of RBC lysate was loaded. The panel on the left shows the Ponceau-stained membrane as loading control. The upper right shows the immunodetection of hPrx-2: hPrx-2 is detected in erythrocyte lysate (ERY) and in extract of *P. falciparum* (PF), but not in FVs (= VAC). Recombinant hPrx-2 was used as a positive control (POS). The lower panel on the right shows the detection of Hb, mainly in the FVs and the erythrocyte lysate, but also in lower amounts in parasite lysate. (Images have been adjusted for contrast and brightness).

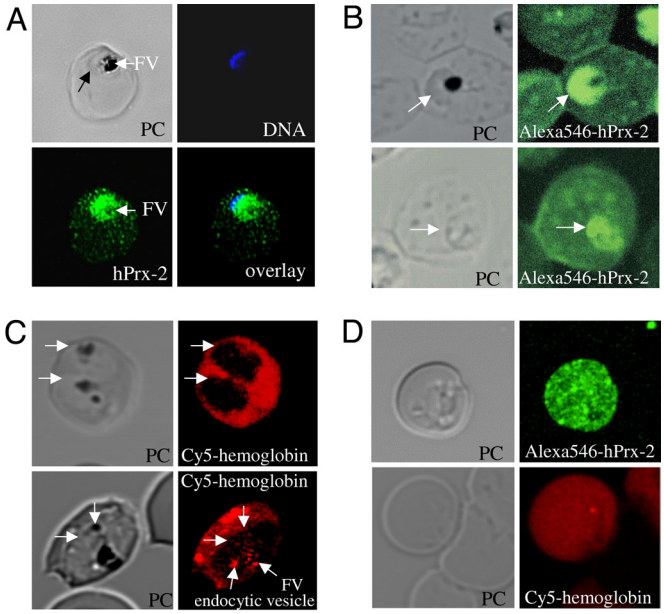

Fig. 2. Confocal laser scanning analyses of hPrx-2 in infected RBCs. (*A*) IFA of hPrx-2 of *P. falciparum* infected RBCs. A polyclonal antibody recognizing the peptide L^{103}LADVTRRLSED^{114} of hPrx-2 was used. Phase contrast (PC) shows the location of the parasite and the food vacuole (FV, *white arrow*) containing hemozoin (*dark pigment*). Black arrow indicates parasite position. (*B*) Confocal laser scanning microscopy of Alexa546-hPrx-2-preloaded RBCs infected with *P. falciparum*. Labeled hPrx-2 is imported into the parasite. (*Upper*) Phase contrast (PC) and fluorescence images of recombinant Alexa546-hPrx-2 preloaded RBCs infected with a trophozoite. The FV is not stained (*dark spot*). (*Lower*) Phase contrast (PC) and fluorescence images of infected RBCs preloaded with recombinant Alexa546-hPrx-2; in that case a late ring-stage parasite is labeled with hPrx-2. White arrows indicate parasite position. (*C*) Confocal laser scanning microscopy analysis of Cy5-Hb-preloaded RBCs uninfected and infected with *P. falciparum* trophozoites. (*Upper* and *Lower*) Cy5-Hb-preloaded (*red*) RBCs infected with *P. falciparum* trophozoites. White arrows indicate parasite positions. The FV and an endocytic vesicle are marked by white arrows and text. (*D*) Confocal laser scanning microscopy analysis of noninfected but fluorescent protein loaded RBCs. (*Upper*) Alexa546-hPrx-2-preloaded RBC. (*Lower*) Cy5-Hb-preloaded RBC.

unit. *P. falciparum* ingests RBC cytoplasm containing Hb through the cytostome, an invagination of the parasite plasma membrane. Hb is then transported through the parasite cytoplasm via vesicular transport and is directed to the food vacuole (FV), where it is digested (7).

The LC-MS results unambiguously show that the relative amount of Hb to hPrx-2 massively shifts toward hPrx-2 in the parasite extract (Table S2). This shift was detected for hPrx-2 when compared to all Hb chains analyzed in the parasite extracts (α, β, and δ). hPrx-2 is enriched at least 10-fold in relation to Hb, indicating a specific uptake of hPrx-2 from the RBC to the parasitic cytosol.

hPrx-2 Is Not Detected in Parasitic FV Preparations. Because hPrx-2 is enriched in relation to Hb in parasite extracts, it is highly unlikely that hPrx-2 is imported to the parasite FV and proteolytically digested. To test this hypothesis, we performed subcellular fractionation of enriched parasitic FVs and remnant cytoplasm (Table S3) (8, 9). Immunoblots showed that hPrx-2 is enriched to the cytosol of *P. falciparum* and is not detected in the FV preparation at used protein loads (Fig. 1*C*). In contrast, Hb is enriched to the FVs. This experiment suggests that the cellular fate of hPrx-2 differs essentially from that of Hb, as the parasite does not take up hPrx-2 to degrade it in the FV.

hPrx-2 Is Imported to the Cytosol of *P. falciparum*. To further study the subcellular localization of hPrx-2 in infected RBCs, we used different imaging techniques. Indirect immunofluorescence analysis (IFA) with confocal microscopy indeed confirmed an accumulation of hPrx-2 in the parasite's cytosol. In contrast, the parasite's FV was not stained (Fig. 2*A*, Fig. S3). In addition, the Movie S1, displaying *z*-axis resolution, shows that the protein is localized inside the parasite cell and not on the surface or in the parasitophorous vacuole. This distribution pattern of hPrx-2 was shown for the strains HB3 [chloroquine- (CQ) sensitive] and Dd2 (CQ-resistant) using 3 different antibodies directed against hPrx-2 (Fig. S4). The active site Cys-51 residue of hPrx-2 is susceptible to "over"-oxidation to sulfinic and sulfonic acid (10). Using an antibody that specifically recognizes these forms, we found the same distribution (see Fig. S4), indicating that the more acidic over-oxidized forms are also present in the parasite. According to their pI-values, these forms could well be represented in the spots 2 to 5 (see Fig. 1).

Uptake of Labeled hPrx-2. As an alternative method to visualize hPrx-2 localization in parasites, we applied hypotonic dialysis loading of RBCs with fluorescence-labeled hPrx-2. Noninfected RBCs were loaded with fluorescent recombinant hPrx-2 (labeled with either Alexa546 or Cy3) (11). These hPrx-2-loaded RBCs were then infected with *P. falciparum* and analyzed by confocal microscopy to assess fluorescent hPrx-2 distribution.

The labeled hPrx-2 was indeed found to be imported into the parasite's cytosol (Fig. 2*B*) and was not detected in the FV (see Fig. 2*A*). The distribution was the same for both Alexa 546 and Cy3 labeling of hPrx-2 (data not shown for Cy3), thus excluding dye-specific artifacts. hPrx-2 was taken up in all parasitic stages observed, as shown for late ring stages (see Fig. 2*B*, *Lower*) and trophozoite stages (see Fig. 2*B*, *Upper*).

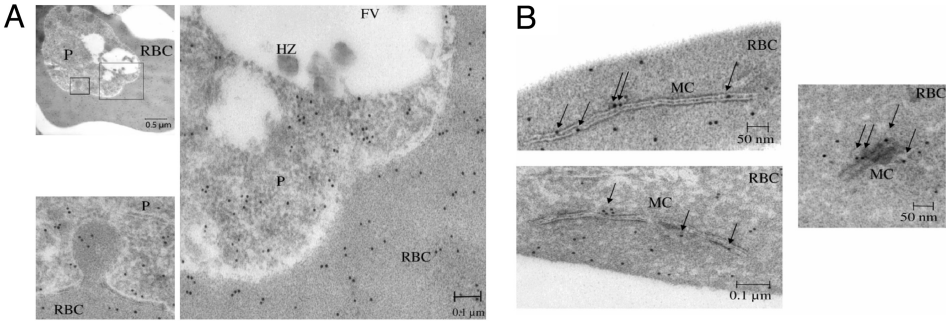

Fig. 3. IEM localization analyses of hPrx-2 in infected RBCs. (*A*) IEM analysis of hPrx-2 of *P. falciparum*-infected RBCs. A polyclonal antibody recognizing the peptide L^{103}LADVTRRLSED^{114} was used. Staining shows an accumulation of the human protein inside the trophozoite cytosol (P) (*black dots* = gold particles). The FV containing residual hemozoin (HZ) is not stained. hPrx-2 staining is also observed in the RBC cytosol and an endocytotic invagination. (*B*) IEM analysis of hPrx-2 of *P. falciparum*-infected RBCs shows the presence of the human protein in and attached to Maurer's clefts (MC).

To check for the protein specificity of the method, similar experiments were performed with fluorescently labeled human Hb. As expected, a completely different fluorescence distribution was observed. Hb, in contrast to hPrx-2, did not accumulate in the parasite cytosol (Fig. 2*C*). The Hb staining pattern is in accordance with current knowledge of Hb metabolism in *Plasmodium* and subcellular localization studies of Hb (12), showing that it is mainly present in the FV. These data confirm that hPrx-2 is imported to the parasite's cytosol and that the observed uptake of labeled hPrx-2 is not artifactual through uptake of free dye, but instead is specific and dependent on the nature of the protein.

EM Substantiates the Presence of hPrx-2 in Parasite Cytosol and Reveals Association with the MCs. To verify the above-described results, we applied EM and immunogold labeling (IEM) of hPrx-2 in infected RBCs. Electron micrographs show an intense staining of hPrx-2 in the parasite cytosol and in endocytic vesicles (Fig. 3*A* and Fig. S5*A*). Again, no hPrx-2 labeling was detected in parasitic FVs. Fig. S5*A* gives another example of hPrx-2 labeling: the section shows that the PVM and the parasitophorous vacuole are not stained.

Relative quantitation of the IEM data of hPrx-2 in infected RBCs shows stronger labeling in the parasite cytoplasm compared to the host cell. Semiquantitative analysis of hPrx-2 labeling in infected RBCs and parasites revealed an enrichment of hPrx-2 by a factor of ≈1.5 to 2 in the parasite (see the example in Fig. S5*B*). These data are supported by the data from the LC-MS experiments. The LC-MS analysis has shown that when the same amounts of the complex cellular protein mixtures are applied, the hPrx-2 is present ≈3-fold higher (quantified as spectrum count, but also reflected in numbers of identified peptides) in parasite extract than in extract from infected RBCs (see Table S2).

Besides proving the presence of hPrx-2 in the parasite cytosol, the IEM revealed a further interesting feature of hPrx-2 topology: hPrx-2 is not only located in the parasite's cytosol but it is also present in or attached to the MCs in infected RBCs (Fig. 3*B*). The observed hPrx-2 staining pattern of MCs is comparable to other published IEM MC stainings (13).

hPrx-2 Colocalizes with Cytosolic GFP in the Parasite Cytosol and with *P. falciparum* Skeleton-Binding Protein-1 in the MCs. Prx require a reducing substrate for enzymatic activity. The reducing substrate of parasitic Prx is thioredoxin 1 (*Pf*Trx1), a cytosolic protein. We used a parasite transfectant expressing cytosolic GFP to perform further IFAs (Fig. 4*B*) (14). The experiments showed that hPrx-2 colocalizes with cytosolic GFP, thus confirming the above-described experiments, which indicate localization to parasite cytosol (Fig. 4*B*). hPrx-2 presence is also shown for late schizont/merozoite stages (Fig. 4*A*).

We were also able to verify the presence of hPrx-2 in the MCs, as already suggested by IEM. IFAs show that hPrx-2 colocalizes with *P. falciparum* skeleton-binding protein 1 (*Pf*SBP1), a marker for the MCs (Fig. 4 *C* and *D*) (15). Fig. S3 displays sections of confocal IFAs that show further examples of hPrx-2 staining the parasite cytosol and MCs.

hPrx-2 Is a Molecular Interaction Partner of, and Enzymatically Active with, Parasitic *Pf*Trx1. To study if hPrx-2 is enzymatically active in the parasitic cytosol, we investigated the direct molecular interactions between hPrx-2 and cytosolic *Pf*Trx1. This interaction was proven by pull-down experiments with parasitic-cell extracts. Similar approaches have previously been used to find interaction partners for thioredoxins (16, 17). A *Pf*Trx1 mutant C33S, lacking the resolving cysteine at its active site, was used to trap interacting proteins via a disulfide bond. With this approach, a 22-kDa band was found to be 1 interaction partner of *Pf*Trx1 (see band 1 in Table S1). The band was identified by MS as hPrx-2.

We verified this proposed interaction of hPrx-2 and *Pf*Trx1 by kinetic analyses with recombinant components of the thioredoxin system of the RBC and the parasite (18). We show that hPrx-2 efficiently uses parasitic *Pf*Trx1 as a substrate. The human and the corresponding parasitic (*Pf*TPx1) Prx catalyze the transfer of electrons from either $hTrx^{C73S}$ or *Pf*Trx1 to the 3 hydroperoxides studied (Table 1). Under given conditions, the reaction velocity with the parasitic proteins is roughly 3 to 4 times higher than for the human proteins. Most importantly, hPrx-2 shows 50 to 60% of its original activity using *Pf*Trx1 instead of $hTrx^{C73S}$ as an electron donor, which proves efficient detoxification of peroxides.

A Significant Proportion of Thioredoxin Peroxidase Activity in Parasite Extracts Is Provided by hPrx-2. Because the RBC is devoid of functional transcriptional and translational machineries, reverse genetics cannot be performed to prove a functional role of hPrx-2 import. Furthermore, specific inhibitors for the different Prx are not available. To circumvent this problem and to pinpoint a functional role of hPrx-2 in the parasite, we depleted hPrx-2 from trophozoite extracts by immunoprecipitation (IP). Next, TPx activity of hPrx-2-depleted extracts was compared to non-depleted controls. Assays with cell lysates were carried out using degassed solutions under argon to prevent over-oxidation of hPrx-2, which usually occurs in the presence of oxygen and cellular substrates. The assays were performed with tert-butylhydroperoxide (tBOOH) as peroxide substrate and reproducibly showed significantly lower activity in hPrx-2-depleted samples (Fig. 5). While control samples had a mean thioredoxin-

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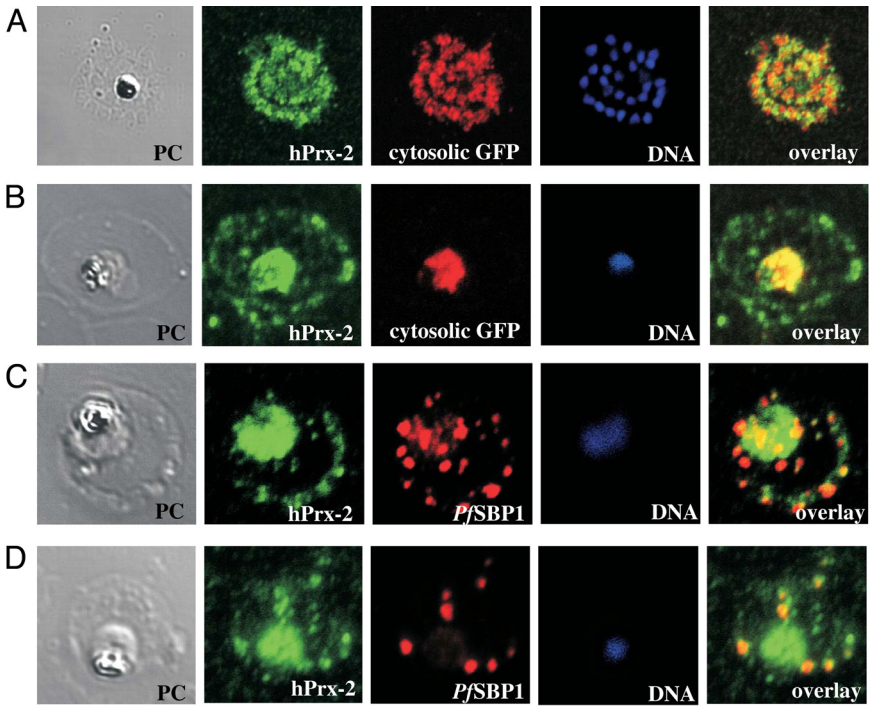

Fig. 4. Confocal laser scanning IFA colocalization analyses of hPrx-2 in infected RBCs. (*A*) IFA colocalization of hPrx-2 with cytosolic GFP in *P. falciparum* late schizont/merozoites. The surrounding RBC is lysed and merozoites are about to be released. Phase contrast (PC) shows the residual hemozoin crystal and merozoites. DNA staining (*blue*) visualizes 28 merozoites. Cytosolic GFP (*red*) stains the merozoites and anti-hPrx-2-staining shows presence of hPrx-2 in merozoites (hPrx-2, *green*). ''Overlay'' shows a weak colocalization of GFP and hPrx-2. (*B*) IFA colocalization analysis of hPrx-2 in a trophozoite. PC shows the parasite and the FV containing hemozoin. Anti-hPrx-2-staining (*green*) reveals the presence of the protein in the trophozoite cytosol as it colocalizes with cytosolic GFP (*red*). (*C* and *D*) IFA colocalization of hPrx-2 with *Pf*SBP1 in *P. falciparum* trophozoites. *Pf*SBP1 (*red*) stains the MCs of the trophozoite. Anti-hPrx-2-staining shows presence of hPrx-2 in parasite cytosol and focal staining in the RBC (hPrx-2, *green*). ''Overlay'' shows colocalization of hPrx-2 and *Pf*SBP1 in the MCs.

dependent peroxidase activity of 20.5 (±3.9) mU/mg of trophozoite protein, the hPrx-2-depleted samples displayed an activity of 11.2 (±4.0) mU/mg. These data imply that the imported hPrx-2 accounts for roughly a half of the overall thioredoxin-dependent peroxidase activity in parasite extract.

CQ Treatment Leads to Raised Levels of hPrx-2 in *P. falciparum*. Our comparative proteomic analyses support the notion that hPrx-2 is taken up and functional in *P. falciparum*. CQ is known to exert oxidative pressure on the parasite by impeding hemozoin formation (19). We have analyzed the response of the CQ-sensitive strains 3D7 and HB3, as well as 2 CQ-resistant strains Dd2 and K1, to CQ pressure at the trophozoite stage. The most eminent and reproducible change in protein abundance after CQ treatment was seen in 1 spot (see Fig. 1, Spot 1). This spot was significantly up-regulated in all 4 strains under CQ pressure (by a factor of 1.6–4.5) (Fig. 6) and was unambiguously identified as hPrx-2 by means of MS.

Because of the reproducibility of these results (using 4 different strains), the presence of hPrx-2 in the extract is unlikely to be caused by a contamination with host-cell material, but rather because of a specific uptake of hPrx-2 to the parasite cytosol, as also suggested by the LC-MS data.

Table 1. Steady-state kinetics of human and parasite peroxiredoxins

		$V/[E]$ [min^{-1}]	
		*Pf*TPx1	hPrx-2
*Pf*Trx1	H_2O_2	48.5	7.7
	tBOOH	34.6	4.1
	Cumene	21.8	4.1
$hTrx^{C73S}$	H_2O_2	35.7	12.7
	tBOOH	28.7	8.6
	Cumene	24.6	7.1

hPrx-2 detoxifies hydroperoxides [hydrogen peroxide, tert-butyl-hydroperoxide (tBOOH), cumene hydroperoxide] using the parasitic *Pf*Trx1 as a reducing substrate. Values represent means of 3 independent experiments, which differed by less than 10%.

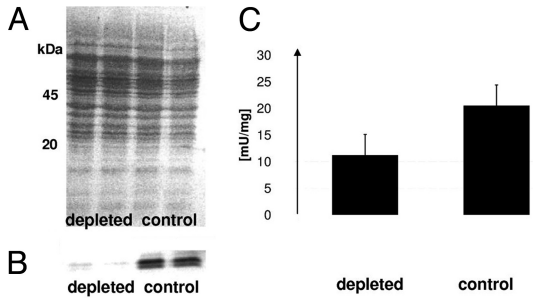

Fig. 5. Depletion of hPrx-2 from parasite extracts. (*A*) Ponceau staining of 75-μg parasite extract. The 2 left lanes represent an hPrx-2-depleted sample, the right lanes 2 undepleted controls. (*B*) Immunostaining of the membrane with anti-hPrx-2 antibody. hPrx-2 was efficiently depleted. (*C*) Thioredoxin-dependent peroxidase activity in parasite lysates. The depletion of hPrx-2 reduces activity with tBOOH to approximately 50% from 20.5 mU/mg parasite protein to 11.2 mU/mg. Data represent the mean of 2 independent cell lysis and depletion experiments and 8 enzymatic measurements.

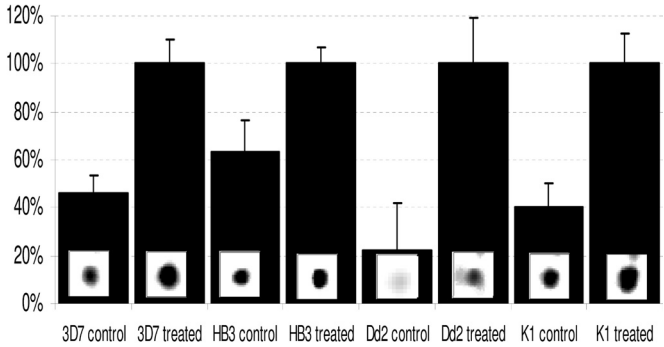

Fig. 6. Regulation of Spot 1 hPrx-2 upon treatment with CQ in 4 *P. falciparum* strains measured by image analysis of 2DE gel spots. Mean spot volumina [intensity × area] were calculated from 3 to 4 measurements. Treated samples were set to 100%. Higher abundance of Spot 1 is seen in all 4 strains after treatment with CQ, thus indicating an enhanced uptake of hPrx-2.

Discussion

Our data demonstrate that the human redox protein peroxiredoxin 2 is imported from the host erythrocyte into the cytosol of *P. falciparum* in functional form and in significant concentrations. This result provides previously undocumented insights into the complex interactions between malarial parasites and their host cells. The fact that the hPrx-2 activity accounts for about 50% of the total thioredoxin peroxidase activity of the parasites substantiates the biological relevance of the data. hPrx-2 had previously been detected on 2DE gels of parasite extracts; however, it was considered a contaminant and not further investigated (20–22). We now prove the presence of hPrx-2 in 4 parasite strains and different life-cycle stages of *Plasmodium*. hPrx-2 mainly localizes to the cytosol of the parasite, where it presumably detoxifies peroxides using *Pf*Trx1 as a reducing substrate. Furthermore, we demonstrate enhanced uptake of hPrx-2 under CQ treatment in all 4 parasite strains, which points to a possible adaptation of Prx import to metabolic needs.

At this stage we can offer 2 working hypotheses that can serve as starting points for further characterizing the exact hPrx-2 import mechanism. (*i*) As shown by different independent methods, hPrx-2 does associate with MCs. This suggests that the MCs, in addition to previously characterized export pathways to the RBC surface (5, 23), could be involved in protein import mechanisms in infected RBCs. In case this hypothesis can be further substantiated in follow-up studies, it would represent a completely unique transport pathway. (*ii*) We furthermore have detected hPrx-2 in endocytic vesicles of the parasite. Thus, a postendocytotic mechanism, exporting hPrx-2 from endocytic vesicles to the parasite cytosol, should be taken into consideration.

In this context, it should be pointed out that the complex protein trafficking mechanisms in the host-malaria parasite unit are still poorly understood. The mechanistic details of parasitophorous vacuolar membrane translocation including signal have only recently received interest as a translocon of exported proteins has been identified (24). Compared to that, an understanding of the mechanisms of functional protein import is at the very beginning. We believe that our results can serve as a solid starting point for studying the uptake and sorting mechanisms of hPrx-2 and for understanding how the parasite differentiates between host material as food supply (targeted to the FV) and adopted host proteins serving the parasite's functional needs. There is accumulating evidence for an import of functional proteins by the parasite. However, the mechanisms of import have not been elucidated up to now and the route of transfer from the RBC to the parasite cytoplasm needs to be further investigated by, for example, pulse chase experiments, serial ultratructural analyses, and uptake inhibition studies. These studies will help to understand basic parasite biology and potentially offer working points for the development of urgently needed new antiparasitic drugs.

An uptake of host proteins by malarial parasites, including δ-aminolevulinic acid dehydratase (25) and ferrochelatase (26), has been suggested previously. Furthermore, catalase from the human host was found to be present and digested in the FV (27) and human superoxide dismutase was discussed to be present in the cytoplasm of the parasite (28). However, other authors had obtained conflicting results, as summarized by Dive et al. (29). It will be of great interest and importance to follow up the cumulating evidence that host cell proteins might be taken up by the parasite to fulfill specific functions.

Based on our data, imported hPrx-2 accounts for ≈50% of the overall thioredoxin peroxidase activity in the parasite. This suggests that hPrx-2 plays a biologically relevant role in the detoxification of hydroperoxides. As previously reported, C-terminally truncated forms of hPrx-2 are enzymatically active, indicating that the lower molecular hPrx-2 forms present in parasite extracts (see Fig. 1) may indeed exert enzymatic activity (30). The second half of the TPx activity measured is attributable to intrinsic parasitic thioredoxin-dependent Prxs, including the cytosolic TPx-1 and 1-Cys Prx. A protective function for hPrx-2 in the parasite is additionally supported by an increase in full-size hPrx-2 in *P. falciparum* under CQ treatment, indicating that the parasite may be able to enhance hPrx-2 import under drug or oxidative pressure. It is therefore tempting to speculate that blocking uptake mechanisms of human proteins into the parasite might represent a promising strategy for future antimalarial drug development.

Instead of depleting its own resources—including energy, substrates, and time—by driving protein biosynthesis, *P. falciparum* has adapted to adopt the hPrx-2, thereby employing the host protein for its own purposes. These findings, addressing the import and function of host proteins, reveal new features of host-parasite interactions and open a field yet to be exploited for parasitological research and antimalarial drug development.

Materials and Methods

Cultivation of *P. falciparum*. Blood stages of the *P. falciparum* strains Dd2 and K1 (CQ-resistant), as well as HB3 and 3D7 (CQ-sensitive), were maintained in culture using standard procedures. Details are described in the *SI Text*.

Preparation of Protein Extracts. Life cycle determination, saponin lysis, parasite lysis, and sample preparation were performed as described in the *SI Text*.

Two-Dimensional Gel Electrophoresis and in Silico Image Analysis. Two-dimensional electrophoreisis was performed according to standardized protocols, as described in the *SI Text*. Gels were stained with silver or coomassie. Scanned gels were analyzed as spot image maps.

Protein Immunoblotting Analysis. Recombinant Prx from *P. falciparum* (*Pf*-TPx1, *Pf*-TPx2, *Pf*-1-Cys-Prx, *Pf*-AOP) were produced as described (18, 31, 32) and used to check anti-hPrx-2-antibody for cross-reactivity in immunoblots. The following polyclonal antibodies were used: rabbit-anti-hPrx-2 recognizing the peptide L^{103}LADVTRRLSED^{114} (Axxora, 1:10,000), rabbit-anti-hPrx-2 recognizing the whole protein, and peroxidase-conjugated anti-rabbit antibody (Dianova, anti-IgG, 1:50,000).

Organellar Fractionation of FVs. Preparation of FVs and residual parasite lysate was achieved according to Jackson, et al. (8), as described in the *SI Text*.

Pull-Down Assays with *Pf*Trx1^{C33S}. Pull-down assays with immobilized thioredoxin and cell lysate were carried out as previously described (16, 17). Briefly, a *Pf*Trx1^{C33S} mutant was heterologously over-expressed, purified, and bound to CNBr-activated Sepharose. *P. falciparum* lysate (≈7–10 mg protein) was incubated with 10 μl of the *Pf*Trx1^{C33S}-immobilized resin for at least 2 h in 100 mM Tris-HCl, 50 mM NaCl, pH 8.0. Unspecifically bound proteins were re-

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moved by washing with the same buffer containing 500 mM NaCl until A_{280} of the eluate was <0.001. The resin was then suspended in elution buffer (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM DTT) for 30 min at 25 °C to elute disulfide bound interaction partners. The eluted proteins were separated by SDS/PAGE, bands excised, and digested with trypsin. Peptides were subjected to PMF analysis.

IP of hPrx-2. Generation of parasite extract and IP were performed with degassed solutions under argon to prevent artifactual overoxidation of hPrx-2 with cellular substrates. For IP, 2 batches of Protein A Sepharose were prepared. One was used to bind polyclonal hPrx-2 antibody and a second batch was incubated with an irrelevant polyclonal rabbit antibody as a control. After 12 h of incubation at 4 °C, Protein A Sepharose-bound antibody was washed with PBS and incubated with parasite extract (1 mg per 100 μl of Sepharose). After 12 h of incubation at 4 °C, supernatants were collected and used for immunoblotting and enzymatic assays.

Protein Identification by PMF with MALDI-MS. Spots and bands of interest were excised from coomassie-stained gels and subjected to tryptic digestion as described (33). For PMF identification, the peptide mass maps were searched against Swiss-Prot (http://www.expasy.uniprot.org) and PlasmoDB (http://www.plasmodb.org) databases by using the search engine Protein Prospector MS-Fit. Standard search parameters were set to allow a mass accuracy of 15 ppm and 2 missed tryptic cleavages.

Cloning and Expression. Recombinant redox proteins from *P. falciparum* (*Pf*Trx1, *Pf*TrxR, *Pf*TPx1) and man ($hTrx^{C73S}$) were produced and purified as described in the *SI Text*.

Enzymatic Thioredoxin Peroxidase Assays. The activities of hPrx-2 and *Pf*TPx1 were measured using thioredoxins from both organisms as reducing substrates for both Prxs, as described in Rahlfs and Becker (18) and in the *SI Text*.

Hypotonic Dialysis for Loading of RBCs with Fluorescence-Labeled Recombinant hPrx-2. Fluorescence-labeled recombinant hPrx-2 was loaded into RBCs by hypotonic dialysis (11). For this purpose, recombinant hPrx-2 was labeled with Cy3 (GE Healthcare). A second batch of hPrx-2 was labeled with Alexa546 (Invitrogen). Human Hb (Sigma) was labeled with Cy5.

Human RBCs of blood group A+ were resuspended to a hematocrit of 50% to a final concentration of 40 μM of the respective labeled protein (hPrx-2-Cy3, hPrx-2-Alexa546, or Hb-Cy5). After dialysis for 90 min against a hypotonic solution (15 mM NaH_2PO_4, 15 mM $NaHCO_3$, 20 mM glucose, 3 mM reduced glutathione, 2 mM ATP; pH 7.4) at 4 °C, an annealing step was introduced (dialysis, PBS, 37 °C, 15 min). Resealing of the RBCs was achieved by adding 9 volumes of 10× concentrated PBS containing 50-mM glucose and a final PBS wash. These loaded RBCs were then infected with enriched trophozoites to reach a parasitemia of 2%. Cells were cultivated as described above for 48 h to the trophozoite stage. For confocal microscopy, labeled, infected RBCs were fixed with 5% methanol/95% acetone and analyzed as described below.

Indirect IFA. Confocal laser scanning IFAs were carried out with *P. falciparum* CQ-resistant (Dd2) and -sensitive (HB3) strains. For the detection of GFP, a transfectant expressing cytosolic GFP was used (14). Cell fixation, antibody incubation, and imaging were performed by standard techniques and microscopic examination using an LSM510 laser scanning confocal microscope (Zeiss Axiovert 100, Carl Zeiss). Details are provided in the *SI Text*.

Electron Microscopy. RBCs infected with *P. falciparum* strain HB3 were fixed with 4% PFA and processed for embedding in LR-White resin, as described (34). Ultrathin sections of LR-white-embedded specimens were prepared and processed for immunogold localization with anti-hPrx-2 antibody (Axxora) (1:500). Secondary antibodies were conjugated to 12-nm colloidal gold. Electron micrographs were taken with a Zeiss EM10 and a Zeiss EM900 transmission electron microscope (Carl Zeiss) operating at 80 kV.

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